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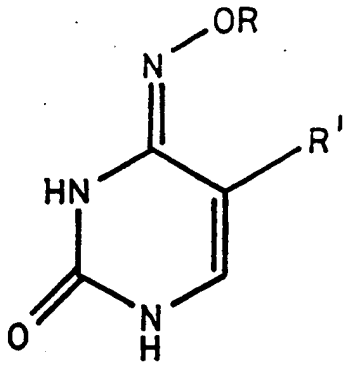

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(57) Abstract

This invention relates to oligonucleotides comprising one or more degenerate base analogues of the structure shown in the figure 4, where R is H or CH_3, R' is H or CH_2OH or R-R' is $-CH_2CH_2-$ forming a cyclic structure, for use as PCR primers and hybridisation probes. Also disclosed are methods of performing PCR and nucleic acid hybridisation, including the use of oligonucleotides containing one or more degenerate base analogues of the present invention.

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Title: Improvements in Oligonucleotide Primers and Probes

Field of the Invention

This invention relates to oligonucleotides and to their use as primers in the Polymerase Chain Reaction (PCR) and as hybridisation probes for the products of PCR.

Background of the Invention

PCR is a widely used technique. It requires the use of oligonucleotides which will hybridise to regions flanking and/or including a particular DNA sequence of interest. This in turn necessitates that the sequence of short sections of DNA flanking and/or including the ends of the region of interest must be determined. Commonly, this is achieved by determining the amino acid sequence of the peptides encoded by the target sequence to be amplified.

However, because of genetic code degeneracy, the nucleotide sequence of the structural gene and the messenger RNA of the organism in which the protein was formed, or a corresponding copy DNA (cDNA), cannot be uniquely defined. Thus the appropriate unique complementary oligonucleotide primers cannot readily be determined. As a hypothetical example, for the following amino acid sequence (1a) the possible messenger RNA (mRNA) (1b) or equivalent cDNA sequences would hybridise best with one of eight possible oligonucleotides (1c).

... met lys his cys... (1a)

... AUG AA^{A}_{G} CA^{U}_{C} UG^{U}_{C}... (1b)

TAC TT^{T}_{C} CT^{A}_{G} AC^{A}_{G} (1c)

Similar problems and considerations apply to the process of probing (by hybridisation) for the nucleic acid sequence encoding a known amino acid sequence.

In practice this problem can be overcome in a number of ways. For instance, multiple primers or probes can be synthesised which correspond to all possible codon assignments. Alternatively, fewer oligonucleotides can be made, based on a codon usage table, allowing for known codon bias of the tissue or organism in question. Additionally, oligonucleotides can be synthesised which incorporate "neutral" bases, such as deoxyinosine (I), which stand in place of A, G, T and C at positions of degeneracy (i.e. those positions where doubt exists over the correct complementary base). However, none of these approaches is ideal. Such methods either require considerable experimental work or have the potential for weakening the hybridisation between primer and DNA sequence under analysis.

Reference 1 discloses a pyrimidine base analogue (capable of binding to A and G with comparable affinity) 3, 4-dihydro-8H-pyrimido [4,5-C] [1,2] oxazino-7-one (known for convenience as P), the structure of which is shown in Figure 1. A deoxyribonucleoside of P, as shown in Figure 2 is also disclosed in this reference. 'Monomers' derived from this deoxyribonucleoside, as shown in Figure 3, which are capable of incorporation into oligomers by automated

DNA synthesisers are also disclosed in this reference.

The use of oligonucleotides containing P bases as hybridisation probes in dot blots and as primers for DNA sequencing reaction has also been described in reference 2.

The present invention is based on the unexpected discovery that oligonucleotides containing synthetic base analogue P and related bases can be used as primers for PCR, and further that such oligonucleotides can surprisingly be more effective as hybridisation probes than conventional multiple oligonucleotides.

Summary of the Invention

According to one aspect of the invention there is provided a method of performing the polymerase chain reaction (PCR), wherein an oligonucleotide used as a primer comprises one or more bases of the structure shown in Figure 4, where R = H or CH_3, R', = H or CH_2OH, or R-R', = $-CH_2CH_2-$ forming a cyclic structure. For brevity, such bases will be referred to as "P and related bases".

In another aspect of the present invention there is provided an oligonucleotide for use as a primer in PCR, wherein said oligonucleotide comprises one or more bases of the structure shown in Figure 4, where R = H or CH_3, R' = H or CH_2OH, or R-R' = $-CH_2CH_2-$ forming a cyclic structure.

P has the structure shown in Figure 4, with RR', being CH_2CH_2 and linking with the rest of the molecule to form a cyclic structure as shown in Figure 1.

The ability to use such oligonucleotides as primers for PCR is unexpected and could not be predicted; DNA sequencing reactions involve the use of DNA polymerases or fragments thereof from the bacterium E. coli, whilst it is an essential element of PCR that the DNA polymerase used is very different, being exceptionally heat-stable. Generally, the enzyme employed for PCR is Taq polymerase, from the extreme thermophile, Thermus aquaticus.

For a base (other than the natural ones) to be capable of use in PCR, primers containing the base must be capable of polymerase chain extension. In addition, in the second phase ("second strand synthesis") the base must be recognised as a base in the extended primer, and there is no reason to expect P and related bases to behave in this way.

Bases of the structure shown in Figure 4 can act as a 'degenerate' pyrimidine analogue in PCR primers, being capable of base-pairing with both A and G with comparable affinity. Use of P and related bases in PCR primers at positions of degeneracy can thus either avoid the need to use multiple primers or reduce significantly the number of different primers required.

Use is conveniently made of PCR primers also incorporating a purine base analogue, capable of binding to both C and T with comparable affinity. For example the base N^6-methoxy-2, 6-diaminopurine (known for convenience as K) as described in reference 3, and related bases may be used for this purpose, as described in the specification of British Patent Application No 9119378.9.

Conveniently, the PCR primer may additionally incorporate a degenerate 'neutral' analogue, capable of base-pairing to both purines and pyrimidines. An example of such an analogue is the compound deoxyinosine (I), which is well known to those skilled in the art.

Oligomers incorporating P and related bases can also be used as hybridisation probes for identifying the products of PCR. Surprisingly probes containing P and another base analogue (particularly I) are found to yield unexpectedly good results in hybridisation experiments when compared to oligonucleotides containing P alone.

In another aspect, therefore, the invention provides an oligonucleotide for use as a probe for the products of PCR, wherein said oligonucleotide comprises one or more bases of the structure shown in Figure 4 where R is H or CH_3, R' is H or CH_2OH, or R-R' is $-CH_2CH_2-$, forming a cyclic structure.

Conveniently, said oligonucleotide further comprises at least one other degenerate analogue. Typically these comprise the analogues I or K, described above.

When used as probes, oligomers according to the invention will generally incorporate a label, in manner well known to those skilled in the art. Such labels may be, e.g., radiolabels, fluorescent labels or enzyme labels.

A further aspect of the invention provides a method of performing nucleic acid hybridisation, comprising the use of an oligonucleotide comprising one or more bases of the structure shown in Figure 4, wherein R is H or CH_3, R' is H or CH_2OH, or R-R' is $-CH_2CH_2-$, forming a cyclic

structure.

Again, typically, such an oligonucleotide will further comprise at least one other degenerate analogue, such as I or K.

Preferably such hybridisation experiments are carried out at temperatures at least 3°C below the predicted T_m for the probe. Typically the temperature is in the range 8-15°C below the predicted T_m.

Primers and probes comprising P and I have been found to give particularly good results.

Clearly, further modifications of P can be envisaged which are within the scope of the present invention.

The invention will be further described by way of illustration and with reference to the accompanying figures in which:

Figure 1 shows the structure at the base known as P;

Figure 2 shows the structure of a deoxyribonucleoside of P;

Figure 3 illustrates production of monomer of P and another monomer K;

Figure 4 shows the general formula P and related bases;

Figures 5 to 9 show the electrophoretic gel separation of PCR-amplified products in which one oligonucleotide primer was the perfect complementary sequence to part of the DNA

sequence to be amplified, whilst the other primer contained either the degenerate bases P, M (M is a base related to P), I or K at one or more positions or was a positive control (i.e. another perfectly complementary primer), and

Figures 10 and 11 show the results of hybridisation (Southern blot) experiments conducted at 45°C or 32°C using multiple oligonucleotides or oligonucleotides containing degenerate analogues.

Synthesis of functionalised controlled pore glass (CPG) support carrying the 3'-O-succinate of (5-dimethoxytrityl-2-deoxyribofuranosyl)-3,4 dihydro-8H-pyrimido [4,5-C][1,2] Oxazin-7-One (2).

P was synthesised as described in reference 1.

As illustrated in Figure 3, the 5'-dimethoxytrityl derivative (1 in Figure 3) of the nucleoside (P) (60mg) was treated in dry pyridine with succinic anhydride (50mg) and 4-dimethylaminopyridine (10mg) for four days. The 3'-O-succinate was purified by chromatography and converted to its 4-nitrophenyl ester by reaction with 4-nitrophenol and dicyclohexylcarbodi-imide. The nitrophenyl ester (45mg) with triethylamine (0.1ml) in dimethylformamide (DMF) was shaken with vacuum-dried aminoalkyl CPG (Pierce Inc.) for 24 hours. The CPG was washed with DMF, ether and dried then treated with acetic anhydride in pyridine for 10 minutes, then washed and dried as before. The nucleoside loading of the functionalised CPG (2 in Figure 3) was 57.2 umol./g.

Oligonucleotide Synthesis

Oligonucleotides were synthesised using an Applied Biosystems Instrument with the normal synthesis cycle. In addition to the normal protected nucleoside-3' (N,N-diisopropyl cyanoethyl) phosphoramidite monomers, the corresponding phosphoramidites of the nucleosides carrying the pyrimidine bases P (3 in Figure 3) and M (M has the structure shown in Figure 4, with R = CH_3 and R' = H see also reference 4), and the purine K (4 in Figure 3) (see reference 3) and also the "neutral" base I were used in the synthesis of oligodeoxyribonucleotides.

In other applications the CPG functionalised with dimethoxytrityl derivative of the nucleoside P was used to provide oligonucleotides having the P nucleoside at the 3'-end of the oligomer.

Oligomers were purified by hplc using an ion-exchange column in the usual way.

Representative oligonucleotide sequences are given in the Figures 5 to 9. These have one or more of the bases P, M and K alone (Figures 5 to 8) P and K together (Figures 7 to 9), or I alone or together with P (Figure 9).

The Use of oligodeoxyribonucleotides containing degenerate bases P, M, I and K as primers in the polymerase chain Reaction (PCR).

The following examples illustrate the usefulness of the present invention. In general, single stranded bacteriophage M13 DNA which contained an insert corresponding to the Tyr Ts gene of B. stearothermophilus (reference 5) was used. The standard PCR protocol was

used and the thermal cycle optimised as described (reference 6). Thus, for PCR, a Techne programmable Dri-Block PHC-1 apparatus was used. Each 100ul reaction contained 1um DNA, 200 pmol. of each primer, 4-5 U of Taq DNA polymerase in the recommended buffer (reference 6). Typical thermal cycles were: denaturing temp. 92°C for 1 min.; annealing temp. 36-44°C for 1 min.; chain extension temp. 62-70°C for 1 min. and the number of cycles was 30. The regions of the gene to be amplified are shown in the Figures. After amplification the products were electrophoretically separated on standard agrose minigels. Representative results are shown in Figures 5 to 9.

In Figures 5 to 9 the sequences of the DNA at which the oligomers prime are shown, together with the primer sequences and the base pairs (e.g. P/A) that the degenerate bases form on hybridisation to the template DNA. Thus P is hybridised to A and to G (as is M) and K is hybridised to T and to C. Fully complementary (perfect) primers are used as positive controls; negative controls have one primer missing. The amplified DNA in each case is shown to have the correct chain length by reference to restriction nuclease HaeIII digest of ØX174 DNA.

Figure 5 shows that a primer with a 3'-terminal degenerate pyrimidine analogue, a P base, (lane 3) can be chain extended by Taq polymerase and that P (lane 6) is more suitable than the M, alternative pyrimidine analogue (lane 5), for inclusion in PCR primers.

Figure 6 shows that a primer containing three P bases (lane 4) is almost as effective at priming chain extension by PCR as the perfectly complementary primer (lane 1),

whilst a primer containing three mis-matched bases (lane 3) is ineffective.

Figure 7 shows that oligomers containing up to three degenerate purine analogue bases (K) are also effective as PCR primers (lane 3) and that oligomers containing both P and K bases (lanes 4, 5 and 6) are effective as PCR primers.

Figure 8 shows that oligomers containing both P and K bases can be used simultaneously as forward and reverse primers (lanes 4 and 6) to achieve PCR amplification.

Figure 9 shows that, whilst oligomers containing 4 'neutral' bases (I) are unsatisfactory PCR primers (lanes 4 and 5), giving rise to more than one product (implying non-specific priming), oligomers containing 2I and 2P bases (lanes 6 and 7) are suitable PCR primers and give results comparable to primers containing 2K and 2P bases (lanes 2 and 3).

It is known from the prior art (reference 2) that oligonucleotides containing P or I can be used as probes in dot blots. Further experiments described herein were conducted to investigate the usefulness of oligonucleotides containing degenerate analogues as hybridisation probes in southern blots.

The amino acid sequence of a protein, part of NADH/ubiquinone oxidoreductase, was determined, as described by Walker et al., (reference 7). The protein is termed ASHI, from the first four amino acids of the N terminal. The N terminal amino acid sequence of ASHI is shown below, using the conventional single letter

notation:

A S H I T K D M L P G P Y P K T P E E R
F P R

The nucleic acid sequence encoding amino acids H(3) to R(20) was amplified by PCR using forward (F) and reverse (R) primers as described in reference 7. The products of the PCR were separated on an agarose gel and blotted onto an inert matrix (Hybond-N, Amersham International, UK) using conventional techniques. These blots were then subjected to hybridisation experiments using oligonucleotide probes complementary to the nucleic acid sequence acid encoding the 6 middle amino acids.

A number of oligonucleotide probes were used. These included probe P, which comprised multiple (1024) oligonucleotides covering all possible codon assignments. The sequence is shown below where R = A and G, Y = C and T, N = A, G, C and T.

P = YTN CCN GGN CCN TAY CC, T_m = 50°C.

The T_m was calculated for each probe by adding 4°C for each G or C base, 2°C for each T or A and nothing for each P, K or I base analogue.

Another probe, P(P + K), comprised a lesser number of mixed oligonucleotides (96) comprising P and K. The sequence is shown below:

P(P + K) = PTN CC^{Y}_{K} GG^{R}_{K} CCN TAP CC T_m = 42°C.

Another probe P(P + I), with lower complexity (48), comprised oligonucleotides containing P and the degenerate 'neutral' analogue I. The sequence is shown below:

P(P+I) = PTN $CC\underset{I}{P}$ $GG\underset{I}{P}$ $CC\underset{I}{Y}$ TAP CC, T_m = 40°C.

Figure 10a shows the results of a Southern blot of PCR-amplified fragments encoding the N terminal amino acid of the ASHI subunit. The blot was probed with probe P at a temperature of 45°C. Faint, hybridising bands of the expected size were observed.

Figure 10b shows the results obtained when the same blot was probed, at the same temperature, with probe P(P + K). The signal returned from the hybridising bands was fainter than that obtained when using probe P. This is presumably because 45°C is above the expected T_m of 42°C.

Figure 11a shows the results yielded by using probe P at a lower temperature (32°C). As expected, the hybridising bands show up more clearly.

Similarly, in Figure 11b, which shows a blot probed with probe P(P + K) at 32°C, the signal from the hybridising bands is stronger. Surprisingly however, the strength of signal is dramatically increased whilst the background is only slightly enhanced.

This dramatic enhancement of the signal: noise ratio is even clearer when using probe P(P + I), as shown by Figure 11c. The hybridising bands are extremely dark whilst there is virtually no background and fewer spurious bands are observed.

The results thus demonstrate that:

a) Solid supports for oligonucleotides functionalised with degenerate bases can be prepared.

b) Oligomers with a degenerate base at the 3'-terminus can act as primers for DNA chain extension and be incorporated.

c) Degenerate bases in oligomers so incorporated are recognised by the polymerase in the second strand synthesis and lead to DNA amplification.

d) Oligomers with several degenerate bases including both purines and pyrimidines are effective primers.

e) Oligomers referred to under (c) and (d) are effective primers when corresponding oligomers forming Watson-Crick mismatches do not lead to amplification.

f) Oligomers comprising degenerate base analogue P may be used as probes for the products of PCR and are surprisingly more effective than conventional mixed probes, especially when comprising at least one further base analogue.

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Claims:

1. A method of performing the polymerase chain reaction (PCR), wherein an oligonucleotide used as primer comprises one or more bases of the structure shown in Figure 4, where R is H or CH_3, R' is H or CH_2OH, or R-R' is $-CH_2CH_2-$ forming a cyclic structure.

2. A method of performing nucleic acid hybridisation, comprising the use of an oligonucleotide comprising one or more bases of the structure shown in Figure 4, where R is H or CH_3, R' is H or CH_2OH, or R-R' is $-CH_2CH_2-$ forming a cyclic structure.

3. A method according to claim 2, wherein hybridisation is performed at a temperature at least 3°C below the predicted T_m for the probe.

4. A method according to claim 2 or 3, wherein said oligonucleotide further comprises at least one purine base analogue.

5. A method according to claim 4, wherein the purine base analogue is N^6-methoxy-2, 6-diaminopurine (hereinafter "K").

6. A method according to any one of preceding claims, wherein said oligonucleotides further comprise a neutral degenerate analogue capable of base-pairing to both purines and pyrimidines.

7. A method according to claim 6, wherein the neutral degenerate analogue is deoxyinosine (hereinafter "I").

8. An oligonucleotide for use as a primer in PCR or for use as a probe for the products of PCR, wherein said oligonucleotide comprises one or more bases of the structure shown in Figure 4, where R is H or CH_3, R' is H or CH_2OH, or R-R' is $-CH_2CH_2-$, forming a cyclic structure.

9. An oligonucleotide according to claim 8, further comprising at least one purine base analogue.

10. An oligonucleotide according to claim 9, wherein said purine base analogue comprises K.

11. An oligonucleotide according to any one of claims 8 to 10, further comprising a neutral degenerate analogue capable of base-pairing to both purines and pyrimidines.

12. An oligonucleotide according to claim 11, wherein the neutral degenerate analogue is I.

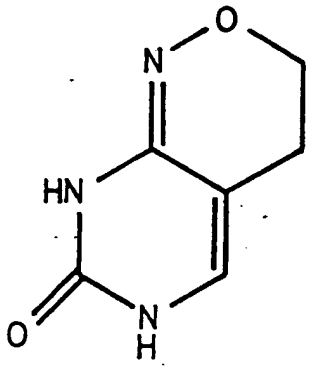


Fig. 1

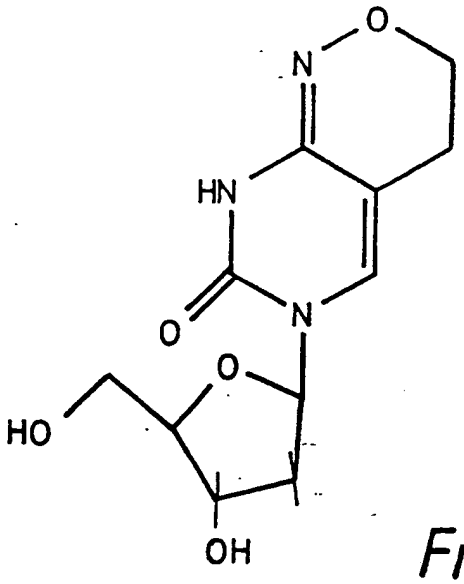


Fig. 2

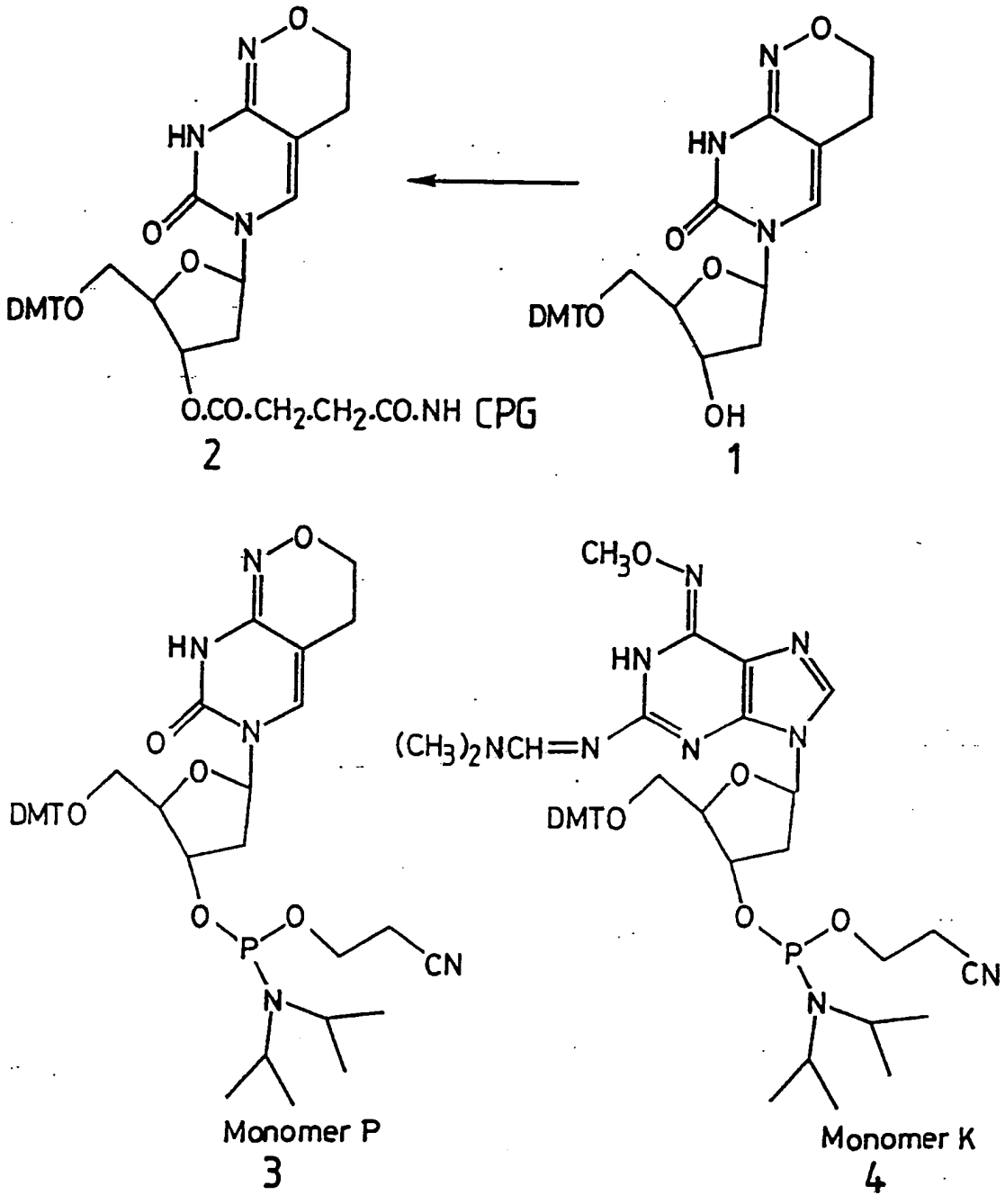

DMTO
O.CO.CH2.CH2.CO.NH CPG
2
DMTO
OH
1
Monomer P
3
(CH3)2NCH=N
DMTO
CN
Monomer K
4

Fig. 3

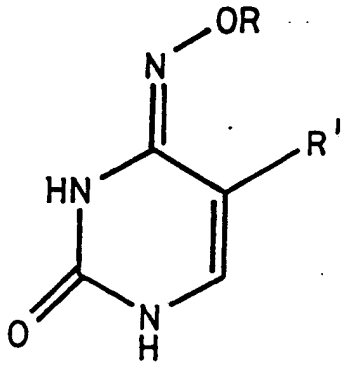


Fig. 4

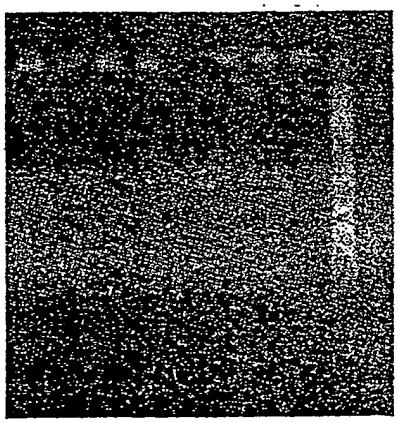
Forward primer **1** 5'-GACGGATGAAGACGGGT-3'

5'----------CAAACGACGGATGAAGACGGGTTGCGCAAACTGTTAAAT----

Reverse Primers

2	3'-TGAA**P**CGGTGG**P**AAAAC-5'	P/G, P/A
3	3'-TGAA**P**CGG**P**GG**P**AAAAC-5'	P/G, P/A, P/A
4	3'-TGAA**M**CGGTGG**M**AAAAC-5'	M/G, M/A
5	3'-TGAACCGGTGGTAAAA**M**-5'	M/G
6	3'-**M**GAACCGGTGGTAAAAC-5'	M/A
7	3'-**P**GAACCGGTGGTAAAAC-5'	P/A
8	3'-TGAACCGG**M**GGTAAAA**M**-5'	M/A, M/G
9	3'-TGAACCGGTGGTAAAAC-5'	PERFECT PRIMER

---------GGCCACTTG**G**GCC**A**CC**A**TTTGACGATGCGCCGCTTCC--------3'



LANE 1 2 3 4 5 6 7 8

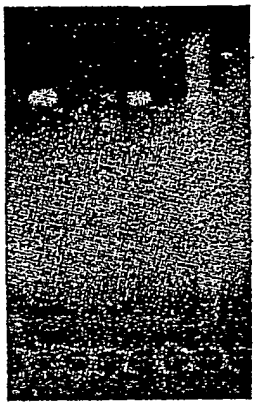
PRIMERS 1 1 1 1 1 1 1 1
5 6 7 8 4 2 3 9

Fig. 5

Forward Primer 1 5'-GACGGATGAAGACGGGT-3' Perfect Primer
5'---CAAACGACGGATGAAGACGGGTTGCGCAAACT-------

Reverse Primers

2 3'-TGAATCGGCGGCAAAAC-5' G/T, A/C, A/C
3 3'-TGAAPCGGPGGPAAAAC-5' G/P, A/P, A/P
4 3'-TGAAMCGGMGGMAAAAC-5' G/M, A/M, A/M
5 3'-TGAACCGGTGGTAAAAC-5' Perfect Primer
---------GGCCACTTGGCCACCATTTTGACGAT---------3'



LANE 1 2 3 4 5
PRIMERS {1 1 1 1 1
{5 4 2 3 –

Fig. 6

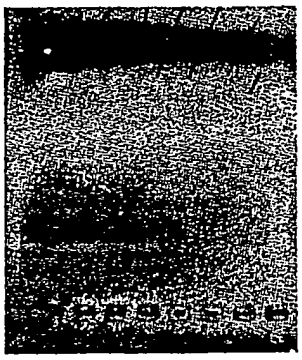
5'----------ACTTGGCCACCATTTTG----------156 BASES

FORWARD PRIMERS

1	5'-ACTTGGCCACCATTTTG-3'	PERFECT PRIMER
2	5'-ACTTGGCCKCCKTTTTG-3'	K/T, K/T
3	5'-ACTTGKCCKCCKTTTTG-3'	K/C, K/T, K/T
4	5'-ACPTGKCCACCKTTPTG-3'	P/A, K/C, K/T, P/ A
5	5'-ACTTGKCCKCPATTPTG-3'	K/C, K/T, P/G, P/A
6	5'-ACPTGKCCACPATTPTG-3'	P/A, K/T, P/G, P/A

----------CGGCCGCTTTTTAGATT----------3'

REVERSE PRIMER 7 3'-GCCGGCGAAAAATCTAA-5' PERFECT PRIMER



LANE 1 2 3 4 5 6 7

PRIMERS { 1 2 3 4 5 6 1 / 7 7 7 7 7 7 - }

Fig. 7

5'---ACTTGGCCACCATTTTG----------

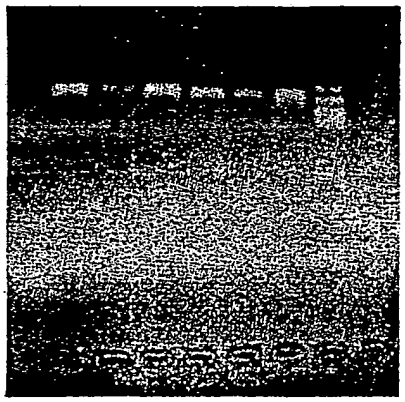
FORWARD PRIMERS

1	5'-ACTTGGCCACCATTTTG-3'	PERFECT PRIMER
2	5'-ACPTGKCCACCKTTPTG-3'	P/A, K/C, K/T, P/A
3	5'-ACTTGKCCKCPATTPTG-3'	K/C, K/T, P/G, P/A

----------GAAGCGGACGGCAATCC----------3'

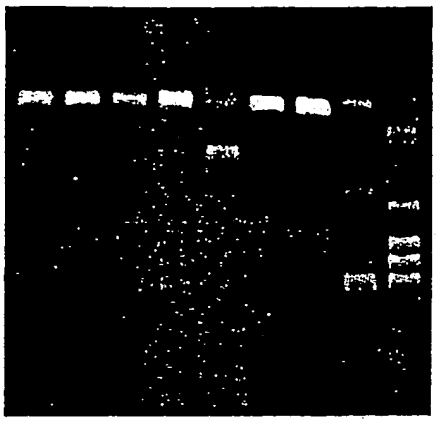
REVERSE PRIMERS

4	3'-CTTCGCCTGCCGTTAGG-5'	PERFECT PRIMER
5	3'-CTPCGPCTKCCGTTKGG-5'	P/A, P/G, K/C, K/T



LANE	1	2	3	4	5	6
PRIMERS	1	1	2	2	3	3
	4	5	4	5	4	5

Fig. 8



PCR EXPERIMENTS

5'--------ACTTGGCCACCATTTTG---------174 BASES----------

Forward Primers

1	5'-ACTTGGCCACCATTTTG-3'	Perfect Primer
2	5'-ACPTGKCCACCKTTPTG-3'	P/A, K/C, K/T, P/A
3	5'-ACTTGKCCKCPATTPTG-3'	K/C, K/T, P/G, P/A
4	5'-ACITGICCACCITTITG-3'	I/A, I/C, I/T, I/A
5	5'-ACTTGICCICIATTITG-3'	I/C, I/T, I/G, I/A
6	5'-ACPTGICCACCITTPTG-3'	P/A, I/C, I/T, P/A
7	5'-ACTTGICCICPATTPTG-3'	I/C, I/T, P/G, P/A

----------GAAGCGGACGGCAATCC----------3'

Reverse Primer

8	3'-CTTCGCCTGCCGTTAGG-5'	Perfect Primer

Fig. 9

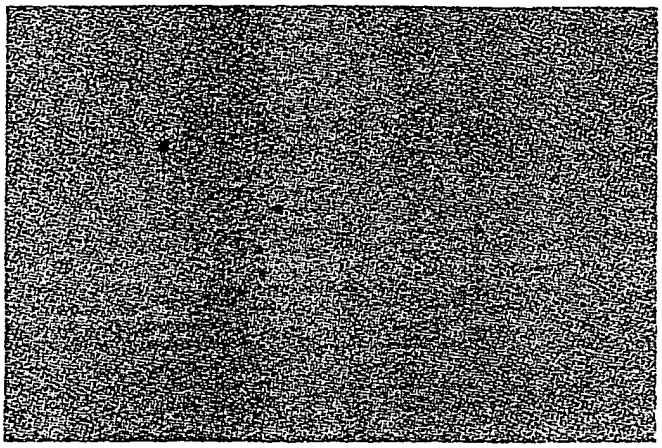


Fig. 10a

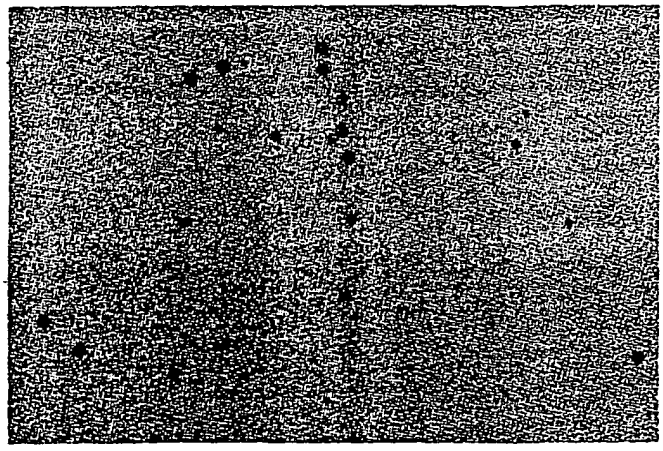


Fig. 10b

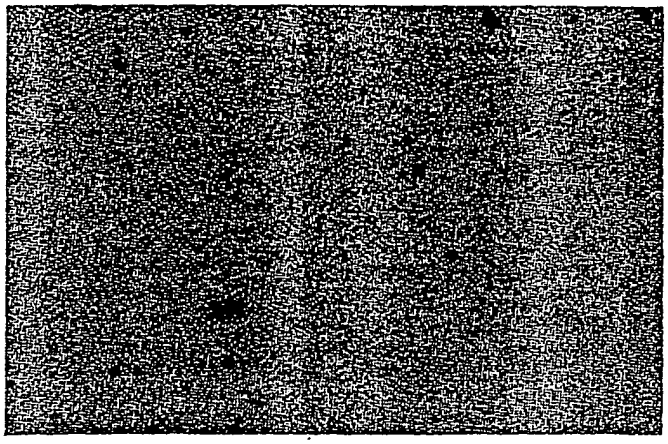


Fig.11a

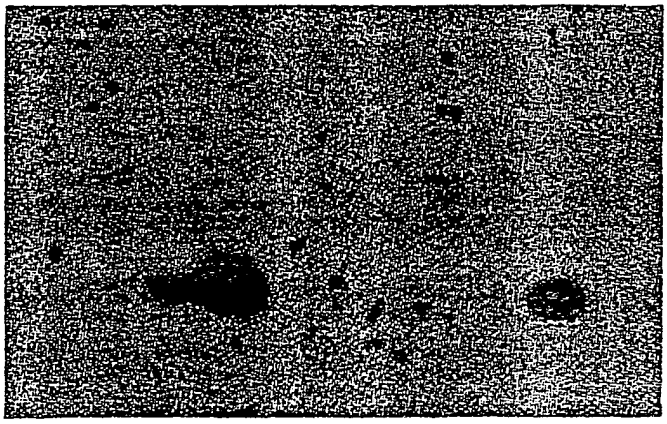


Fig.11b

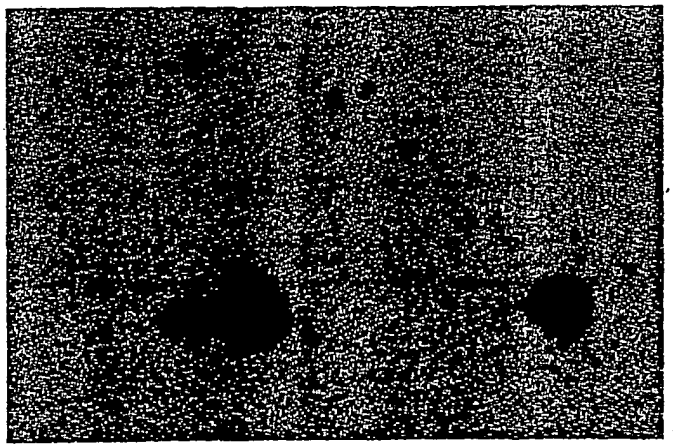


Fig.11c

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/01661

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68; C07H21/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	NUCLEIC ACIDS RES., 17(24), 10373-83 1989, ARLINGTON, VIRGINIA US LIN, P. KONG THOO ET AL. 'Synthesis and duplex stability of oligonucleotides containing cytosine-thymine analogs' cited in the application see the whole document	1-12
X	NUCLEIC ACIDS RESEARCH. vol. 15, no. 20, 1987, ARLINGTON, VIRGINIA US pages 8167 - 8176 N.N. ANAND ET AL. 'The stability of oligodeoxyribonucleotide duplexes containing degenerate bases' cited in the application see the whole document	1-12

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14 DECEMBER 1992	13.01.93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MOLINA GALAN E.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	FEBS LETTERS. vol. 264, no. 2, May 1990, AMSTERDAM NL pages 193 - 197 G. C. HUANG ET AL. 'Molecular cloning of a human thyrotropin receptor cDNA fragment' see abstract	7,12
A	EP,A,0 177 382 (INSTITUT PASTEUR) 9 April 1986	
A	WO,A,9 003 443 (CETUS CORP.) 5 April 1990	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9201661 SA 64374

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 14/12/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0177382	09-04-86	FR-A- 2569406	28-02-86
		JP-A- 61069789	10-04-86
WO-A-9003443	05-04-90	US-A- 5091310	25-02-92
		AU-A- 4337089	18-04-90
		US-A- 5142033	25-08-92